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NKX3.1 is a homeoprotein with prostate-specific expression in adults. Loss of NKX3.1 correlates with prostate cancer progression. Loss of heterozygosity affects NKX3.1 in about 80% of prostate cancers. This project focuses on DNA methylation of the NKX3.1 gene promoter in prostate cancer cell lines and tissues. DNA was analyzed by methylation-specific PCR and sequencing of bisulfite-treated DNA. We also treated cultured cells with the methylation inhibitor 5-azacytidine and the histone deacetylase inhibitor, trichostatin A. There was no effect of 5-azacytidine or trichostatin A on NKX3.1 expression in cultured cell. By bisulfite modified DNA sequencing, we identified some methylated or partial methylated CpG islands in -1056 to 1172 of NKX3.1 gene. Three CpG sites at -921, -903 and -47 wer selectively methylated in different prostate cancer cell lines and were also methylated in some prostate cancer tissue. Methylation in tissue correlated inversely with NKX3.1 protein expression. We also study the effect of Sp1 and Sp3 transcription factors on the expression of NKX3.1, our results showed Sp1 and Sp3 did not influence on the expression of NKX3.1.

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Introduction

NKX3.1 is an androgen-regulated NK-class homeobox gene with expression in both adult mice and humans restricted primarily in the prostate gland (1-5). The human *NKX3.1* gene has been mapped to chromosome 8p21 (6), a locus deleted in 85% of prostate cancer (7-9). Fine-structure mapping and sequencing of the minimally deleted region of 8p21 has placed *NKX3.1* in the center of the 8p21 deletion (10). However, the contralateral allele does not undergo somatic mutation in prostate cancer, suggesting that loss of a single allele may be important in prostate carcinogenesis (6). Consistent with this notion is the finding that heterozygosity for loss of *Nkx3.1* in mice confers prostatic dedifferentiation and hyperplasia suggesting that *NKX3.1* haploinsufficiency is dominant and explaining how *NKX3.1* may have a gatekeeper role in the 85% of prostate cancer cases in which the gene is deleted (4,10,11).

NKX3.1 has a complex function that includes binding directly to DNA via the homeodomain resulting, most commonly, in transcriptional suppression. NKX3.1 also binds to other transcription factors such as serum response factor, also via the NKX3.1 homeodomain. In this case NKX3.1 enhances transcription of SRF-responsive genes. As shown by deletion analysis, access to the NKX3.1 homeodomain by SRF is controlled by interaction of the N-and C-terminal ends of NKX3.1. Deletion of either the N- or C-terminal regions of the protein markedly increases the capacity to coactivate SRF.

The past year we have made substantial progress on the project and have, for practical purposes, fulfilled the goals of the proposal. We have defined the scope of *NKX3.1* methylation and determined gene methylation patterns in cell lines and neoplastic tissues. This annual report will first outline the data as they address the State of Work.

A. Analyze DNA methylation of CpG islands in the NKX3.1 5' upstream region and first exon HpaII/MspI analysis of prostate cancer cell line DNA. Use cell line DNA to develop a methylation-specific PCR assay for tissues. (Year 1)

1. HpaII/MspI digestion and Southern blotting of prostate cancer cell line DNA.

There are numerous CCGG sites located upstream and in the *NKX3.1* gene. Both *HpaII* and *MspI* can recognize CCGG site, but only *MspI* can cleave the sequence when the internal C residue is methylated. We isolated genomic DNA from prostate cancer cell lines by using Puregene DNA isolation Kit (Gentra Systems, Minneapolis, USA) with standard protocol. DNA (25μg) from different cell lines were digested with *HpaII* or *MspI* at 37 °C for 16 hours, then inactivate the enzymes by incubate at 65 °C 30 min. The digested products were run on 2% agarose gel over night, transferred to Zeta-Probe Blotting Membrane (BIO-RAD, Calif), hybridized with an *NKX3.1*-specific probe in Rapid-hyb buffer (Amersham life Science, NJ) according the standard protocol provided by the manufacturer. After hybridization, X-film was exposed on the membrane at –70 °C for 48 hours. The results showed that there were no differences in *HpaII/MspI* digestion patterns between LNCaP, DU-145 and PC-3 prostate cancer cell lines (data not shown), it may because that the CpG islands in NKX3.1 are not fully methylated, but partially methylated as our data indicated below.

2. Synthesis of primers for methylation-specific PCR. Testing of primers on cell line DNA to compare NKX3.1 expressing and nonexpressing cell lines.

To determine primers for methylation-specific PCR, we used bisulfite-modified DNA sequencing method to look for the methylation status of NKX3.1. DNA (2μg) in a volume of 50μl was denatured in 0.3M NaOH at 42°C for 10min. Thirty μL of freshly prepared 10mM hydroquinone and 520μl of 3M sodium bisulfite (Sigma) at pH5.5 were added and mixed. The

samples were covered with mineral oil and incubated at 50°C for 16hr. Modified DNA was desalted with the Wizard DNA clean-up system (Promega) according to the protocol provided by the manufacturer and resolved in 50µl sterile water. 5.5µl 3M NaOH was added into 50µl modified DNA solution and incubated at 37°C for 10min for denaturation, followed by ethanol precipitation with See DNA (Amersham Pharmacia Biotech) as coprecipitant. The DNA was resuspended in 20µl sterile water, stored at –20°C until use. Two µg bisulfite-modified DNA (about 100ng DNA before modification) were amplified by common or nested PCR with *NKX 3.1*-specific primers. Genomic sequencing of bisulfite-modified DNA was accomplished using purified PCR products as template. By this method, we identified partially methylated sites in the *NKX3.1* gene. The results of methylated or partially methylated regions are summarized in Figure 1A and examples of sequencing at selected sites are shown in Figure 1B. The regions were focused from –1056 to 1172 in NKX 3.1 gene, including 5' flanking region, exon I and intron I. The CpG island methylation in this region was higher in PC-3 and DU-145 cells than in LNCaP cells, consistent with the expression profile of *NKX3.1* mRNA (2). Based on the data of bisulfite modified DNA sequencing, primers for methylation-specific PCR were determined and tested with prostate cancer cell lines and prostate tissues.

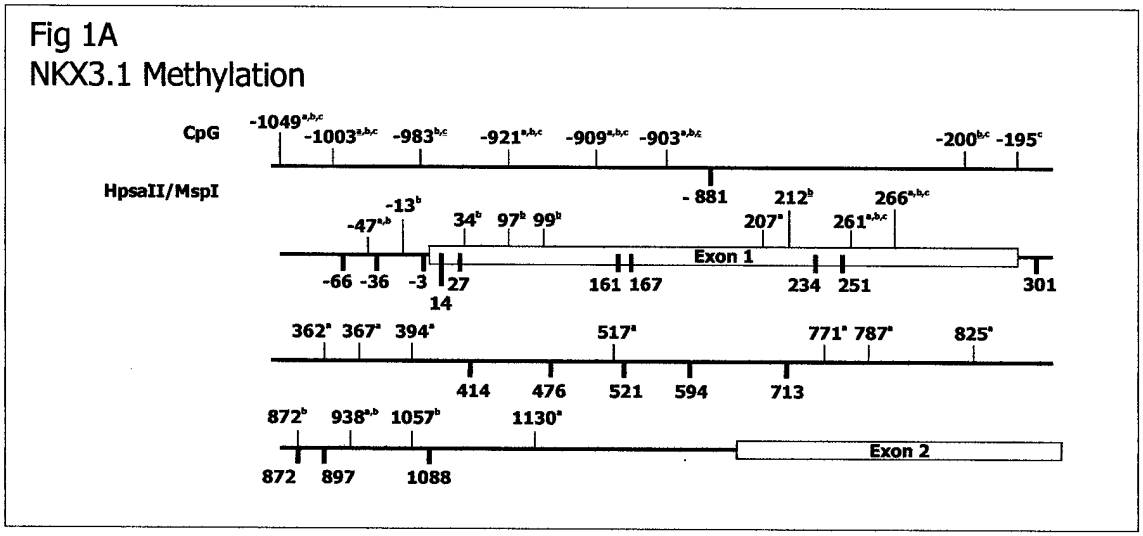


Fig 1A. Methylated CpG dinucleotides of NKX3.1 in prostate cancer cell lines. a – PC3; b – DU145; c – LNCaP; underline – partially methylated. The numbers on the lower portion of the map show locations of CCGG sites.

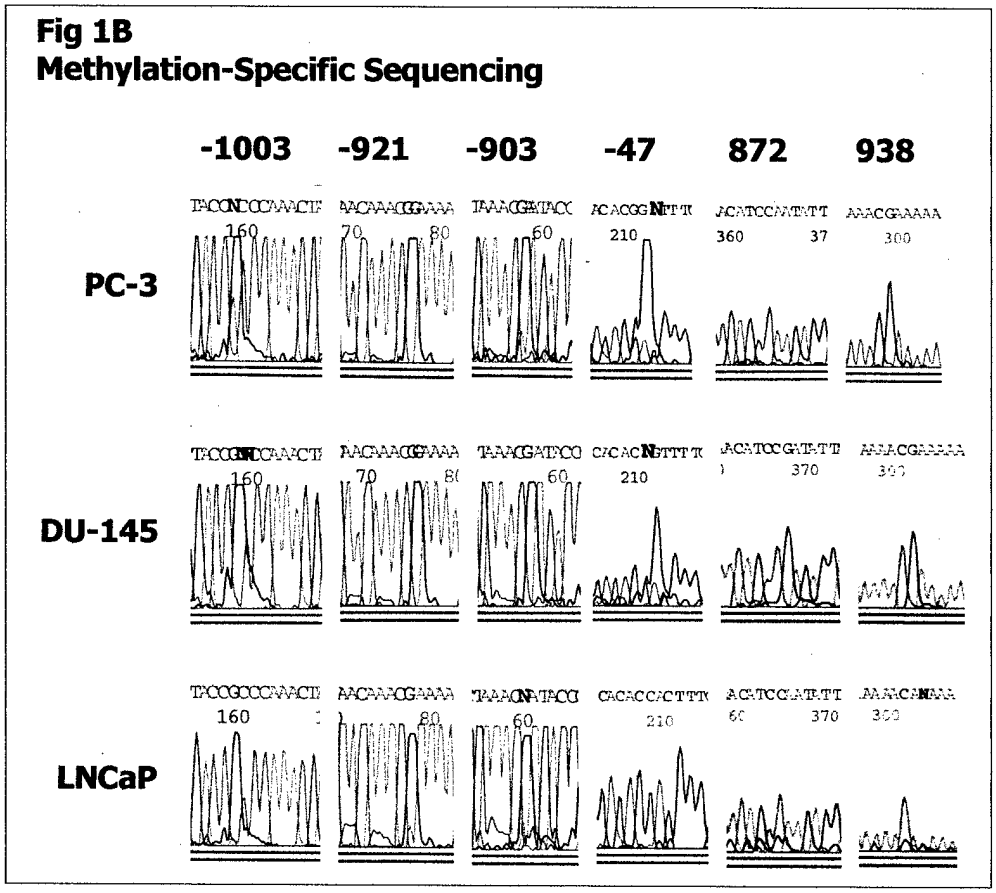


Fig 1B – Methylation-specific sequencing showing methylated CpG sites in three prostate cancer cell lines.

B. Examine promoter methylation in prostate cancer tissues with known expression levels of NKX3.1. Isolate nonmalignant and malignant prostate epithelial cells by laser capture microdissection (LCM) and analyze for NKX3.1 gene methylation by methylation-specific PCR. (Year 2)

1. Extraction of tissue DNA and analysis by methylation-specific PCR

Paraffin blocks were marked for regions of prostate cancer and nonmalignant tissue and microdissected for extraction of DNA. DNA was extracted with QIAamp DNA Mini Kit after removing paraffin wax by xylene extraction. DNA products were modified with bisulfite as above, then used for methylation-specific PCR with specific primers. Figure 2A shows the methylation-specific PCR results of two prostate blocks for the CG islands at sites –921, -903 and -47 of NKX3.1. The primers for 1st PCR of sites –921 and -903 were sense: 5'- GGT ATT TTG AGA GGT TAA GGT AGG AGG ATT -3' and antisense: 5'- CCT ATT AAC TTT CCT TCC TCC CCA AAC ACA TA -3'; the primers for 2nd PCR of site –921 are sense: 5'- GAG GTT GTA GTG AGT TAT GAT GGT -3' and antisense: 5'- CTA TAA CTA AAC TAA ACA ATA CCA TAA CAA CAA ACA -3 (for nonmethylated DNA) or 5'- CTA AAC TAA ACG ATA CCG TAA CAA CAA ACG -3' (for methylated DNA). the primers for 2nd PCR of site –903 are sense: 5'- AGG AAA TCG AGG TTG TAG TGA GTT ATG A -3' and antisense: 5'- TAA TCC AAC CGA TAC TAT AAC TAA ACT AAA CA -3' (for non methylated DNA) or 5'- TAA TCC AAC CGA TAC TAT AAC TAA ACT AAA CG -3' (for methylation status). The primers for 1st PCR of sites –47 were sense: 5'- AGA AGG AGA GGA AAT TGG GGA AGG A –3' and antisense: 5'- CCT CCC TCT AAC TCT AAC TCT AAC TCC -3'; the primers for 2nd PCR are sense: 5'- AGG GAA TTG GGG AAG GAG AGG GAA T -3' and antisense: 5'- CAC CCA CCC AAC CCA CAC CA -3' (for non methylated DNA) 5'- CCC GCC CGA CCC GCA CCG -3' (for methylated DNA). PCR conditions for 1st PCR is 95 °C 15 min; 94 °C 1min, 52 °C 1 min, 72 °C 45s, 35 cycles; 72 °C 10 min, then

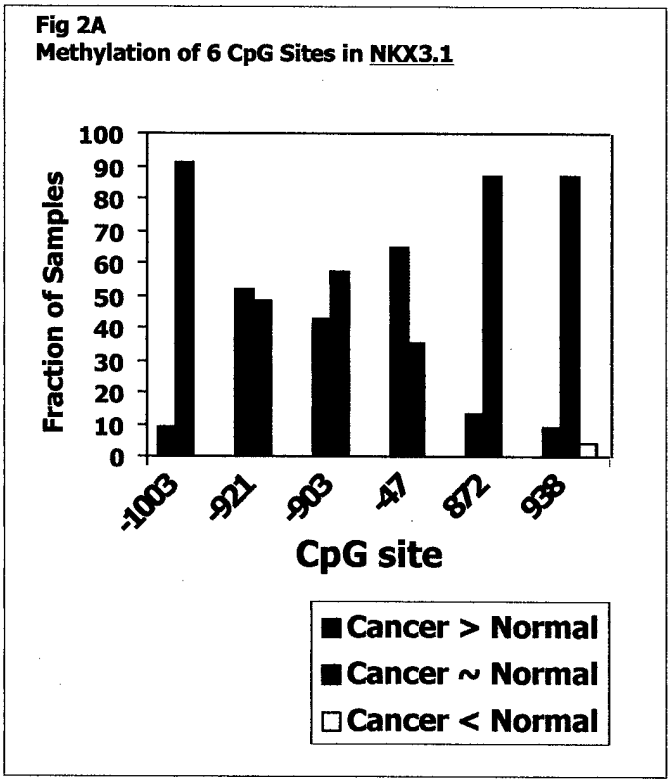
keep at 4 °C. One twenty 1st PCR product was taken as template for 2nd PCR. PCR conditions for 2nd PCR is 95 °C 15 min; 94 °C 1min, 53 °C 45s, 72 °C 35s, 23-32 cycles; 72 °C 10 min, then keep at 4 °C. PCR products were run on 1.6% agarose gel in 1 x TBE buffer with EB (0.4 μg/ml). The target bands were visualized on UV light and take picture by Kodak digital science 1D. From these preliminary data, we can see that methylation of CpG at –921 of *NKX3.1* was higher in cancer tissues compared with normal in the same patient. For this reason this and similar sites were selected for analysis in a panel of tissues.

2. Laser capture microdissection of tissues to assess degree of methylation in cancers that either do or do not express NKX3.1.

Since 90% of DNA was lost after bisulfite treatment (12) we chose not to proceed with laser capture in favor of selecting larger sections of the paraffin block by well-delineated microdissection of cancer and normal cells.

3. Analysis of samples for correlation with Gleason score.

Six CpG sites at -1103, -921, -903, -47, 872 and 938 were analyzed in 40 human prostate cancer specimens by methylation-specific PCR (MSPCR). The primers for 1st PCR of sites 1003 were sense: 5'- GGT ATT TTG AGA GGT TAA GGT AGG AGG ATT -3' and antisense: 5'- CCT ATT AAC TTT CCT TCC TCC CCA AAC ACA TA -3'; the primers for 2nd PCR of site –921 are sense5'- TGA GTT ATG ATG GTA TTA TTG TAT TTT AGT TTG GGC -3' (for nonmethylated DNA) or 5'- TGA GTT ATG ATG GTA TTA TTG TAT TTT AGT TTG GGT -3' (for methylated DNA) and antisense: 5'- CAA ATT ACC AAC TAT TAA CAT ATA ACC CAT -3'. The primers for 1st PCR of sites 872 and 938 were sense: 5'- AGT GAT AAA GTA GGG GTT GAT TAG T -3' and antisense: 5'- CAT TCA TTT ACT AAA AAT CAT CAA AAA CCC -3'; the primers for 2nd PCR of site 872 are sense: 5'- AGT GAT AAA GTA GGG GTT GAT TAG T -3" and antisense: 5'- CCC AAA AAA ACC AAA CCA AAA AAC ATC CA -3' (for nonmethylated DNA) or 5'- CCC AAA AAA ACC AAA CCA AAA AAC ATC CG –3' (for methylated DNA). The primers for the 2nd PCR of 938 are sense: 5'- GAA GAG GGA ATT AAA GTT TAG AAT GAG T -3' and antisense: 5'- TAT AAA AAT TCT AAA TCT ATA TAC TAA AAA AAA ACG -3' (for nonmethylated DNA) 5'- TAT AAA AAT TCT AAA TCT ATA TAC TAA AAA AAA ACA -3' (for methylated DNA). Primers for –921, -903 and –47 are detailed above.



We found that there was uniform methylation of CpG sites in cancer and normal cells at –1003, 872, and 938. On the other hand, -921, -903, and –47 had a substantial fraction of cases where DNA methylation in cancer cells exceeded methylation in normal cells (Figure 2A). An example of these results is shown in Figure 2B. In the top panels one can see that the three sites are methylated equally in normal (N) and cancer (C) cells. In the bottom panels CpG –47 and –921 have darker bands in the "M" lanes indicated methylated DNA compared to the "U" lanes for unmethylated DNA. There is no appreciable difference in methylation at –903 in this tissue

sample. In work that was supported by other funding and not part of the Statement of Work we demonstrated that both gene methylation and *NKX3.1* deletion affected the level of NKX3.1 expression in cancer cells compared to adjacent normal epithelial cells. Figure 2C shows the relative levels of NKX3.1 expression in 48 cancer tissues with information about *NKX3.1* gene deletion and methylation. As can be seen methylation cooperates with gene deletion to reduce NKX3.1 expression below the median for all cancer samples tested.

C. Activate expression of NKX3.1 in prostate cancer cell lines using chemical inhibitors of DNA methyltransferase (5-azacytidine) and histone deacetylase (trichostatin A). (Year 1-2)

1. Treatment of LNCaP, PC-3, DU-145 and TSU-Pr1 prostate cancer cell lines with 5-azacytidine and/or trichostatin A. *Analyze for NKX3.1 mRNA and protein expression. Analyze for change in methylation.*

Fig 2B
Examples of Methylation Data from Prostate Cancer Tissues

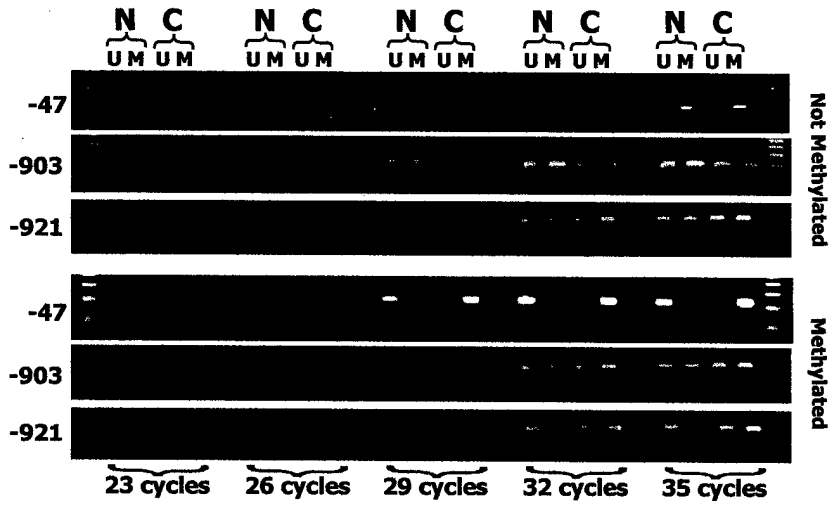
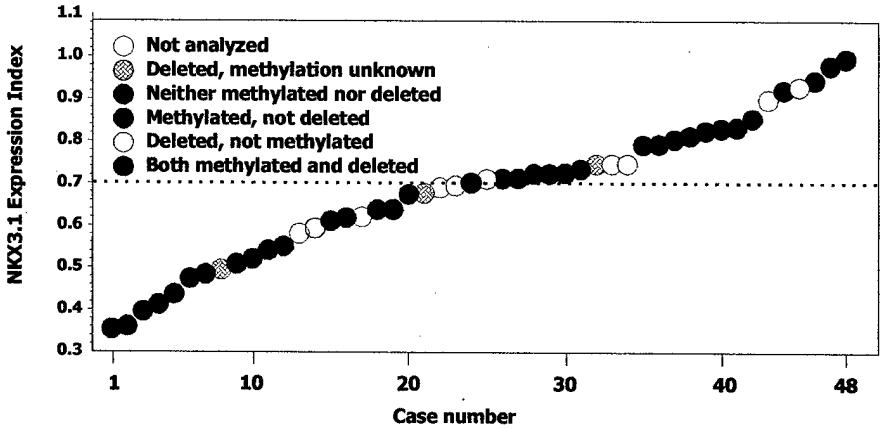


Fig 2C
Methylation, Deletion and NKX3.1 Expression in Prostate Cancer Tissues



The inhibitors of DNA methyltransferase (5-azacytidine) and histone deacetylase (trichostatin A) are commonly used to study the relationship between DNA methylation and gene expression in cells (12). To determine whether control of NKX3.1 expression in cancer cell lines and tissues depended on gene methylation we treated cultured cells with methylation and histone deacetylase inhibitors and assayed *NKX3.1* mRNA expression by RT-PCR. We also analyzed protein expression by western blotting. Prostate cancer cell lines PC-3, DU-145 and LNCaP were cultured in modified IMEM (GIBCO) with 5% fetal bovine serum at 37°C with 5% CO_2 atmosphere. To test inactivation and reactivation, cells were seeded at 2×10^5cells/T75 flask on day 0. After 24 hours, cells were treated with 5-aza-2'-deoxycytidine (5-AC) (Sigma) at a final concentration of 2μM, or with trichostatin A (TSA) (Wako) at a final concentration 200ng/ml, or with both 5-AC and TSA for 24 to 72 hours, exchanging the medium every 24 hours. Identical volumes of solvent alone were used as controls. Total RNA was extracted by RNeasy Mini Kit (Qiagen Inc.Valencia, CA) and treated with DNase (Qiagen Inc.Valencia, CA) to limit the DNA contamination. The

purified RNA was stored at –20°C until use.

RT-PCR primer sequence for NKX3.1 were sense 5'agc cag agc cag agc cag agg 3', antisense: 5' ttg ggt ctc cgt gag ctt gag gtt 3'; RT-PCR product is 332bp. RT-PCR reaction was done with One-step RT-PCR Kit (Qiagen Inc.Valencia, CA). The RT-PCR conditions are as follows: 50°C 30min for reverse transcription, 95°C 15min for initial PCR activation step, then 95°C 1 min, 55°C 1min, 72°C 1min for 30 cycles. RT-PCR products were run on 1.6% agarose gel in 1xTBE buffer with EB (0.4 μg/ml). The target bands were visualized on UV light and take picture by Kodak digital science 1D. Figure 3 and figure 4 shows the results of these experiments. We also found no effects of 5-AC and TSA on NKX3.1 protein expression by western blotting in the three cell lines (data not shown).

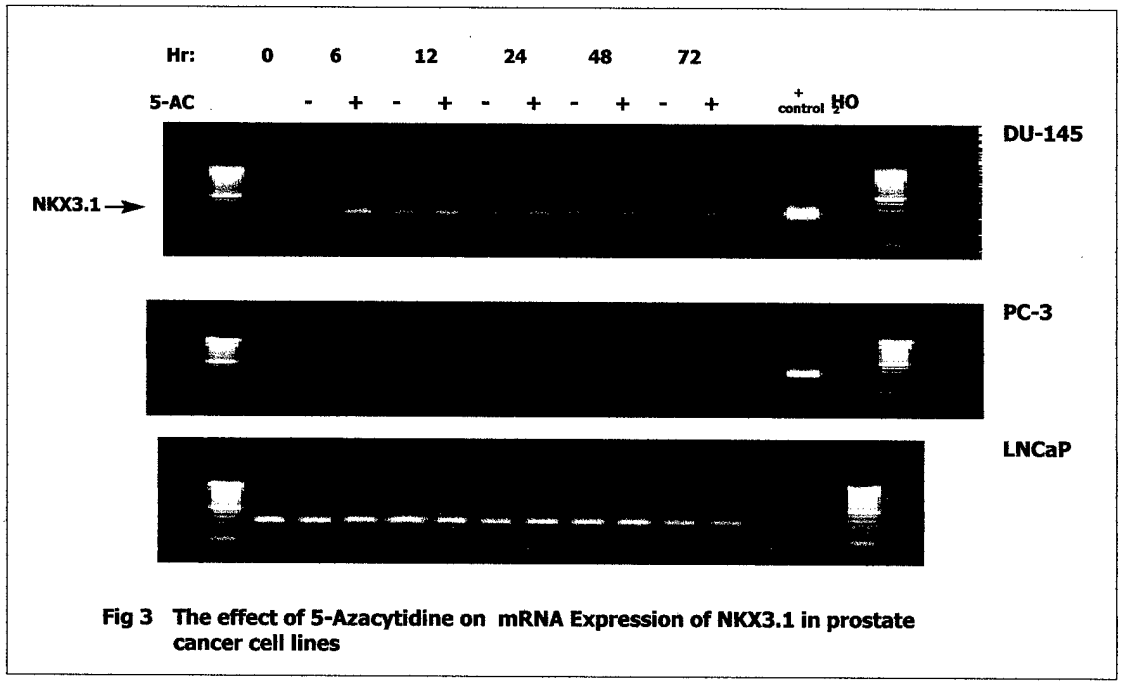


Fig 3 The effect of 5-Azacytidine on mRNA Expression of NKX3.1 in prostate cancer cell lines

To be sure that the 5-AC and TSA were effective, we analyzed expression of androgen receptor (AR) in DU-145 cells after treatment with these agents. Figure 5 shows that consistent with published reports (5) we were able to activate *AR* expression in DU-145 cells. Based on these results, there was no effect of 5-AC and TSA on *NKX3.1* expression in these prostate cancer cell lines. The analysis of *NKX3.1* methylation status in prostate cancer cell lines after treatment by inhibitors of methylation and histone deacetylase was not addressed.

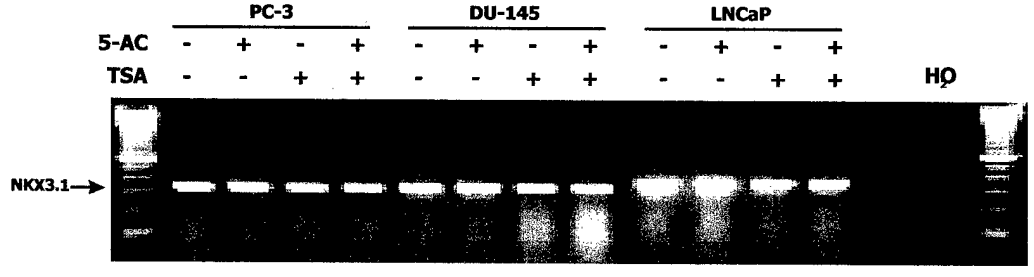


Fig 4 The effect of 5-Azacytidine and Trichostatin A on mRNA Expression of NKX3.1in prostate cancer cell lines. Culture time was 24 hours

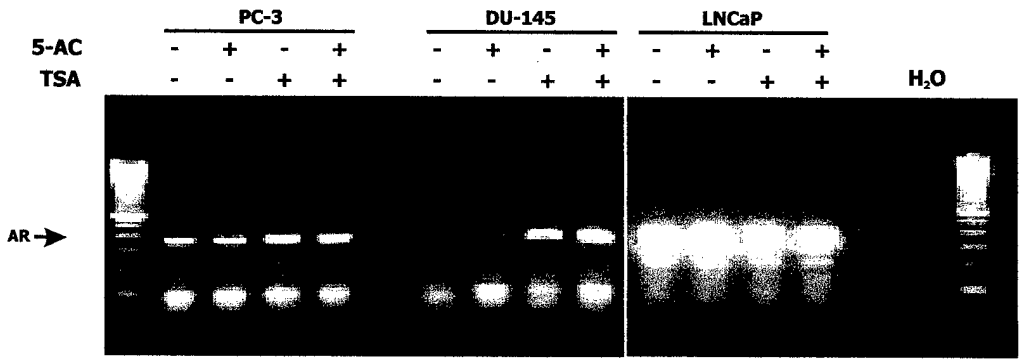
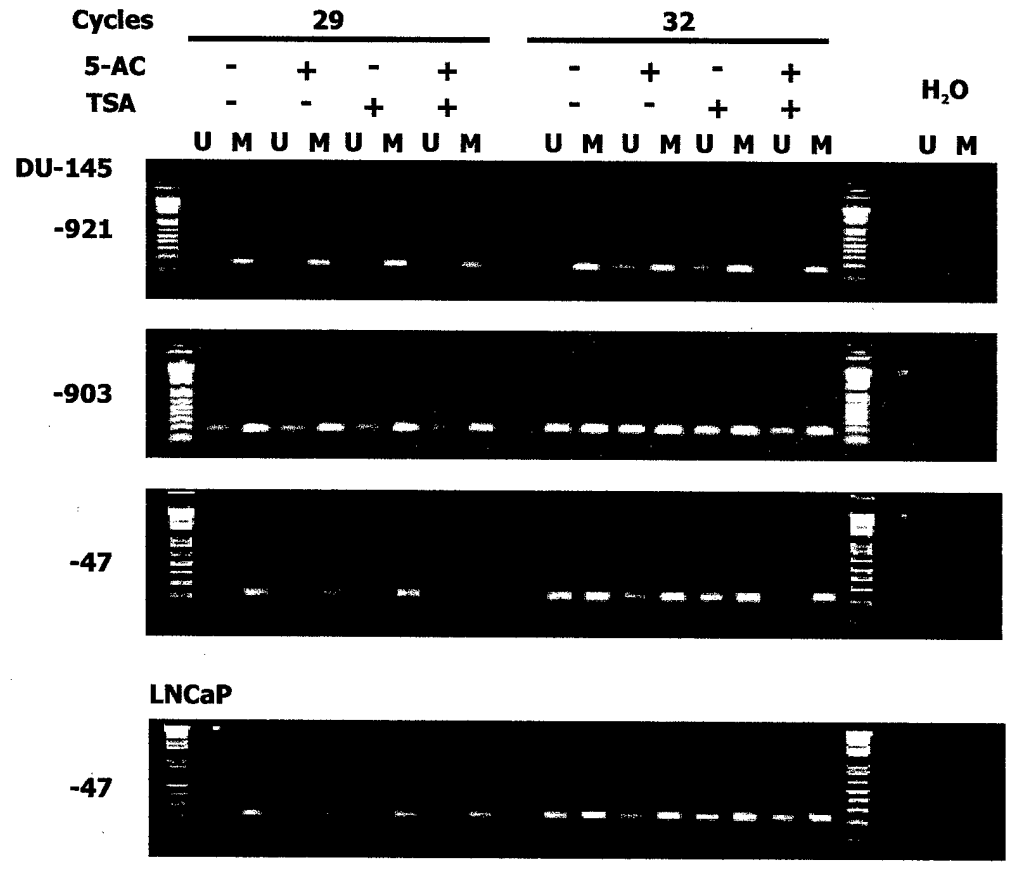


Fig 5 The effect of 5-Azacytidine and Trichostatin A on Androgen Receptor mRNA Expression

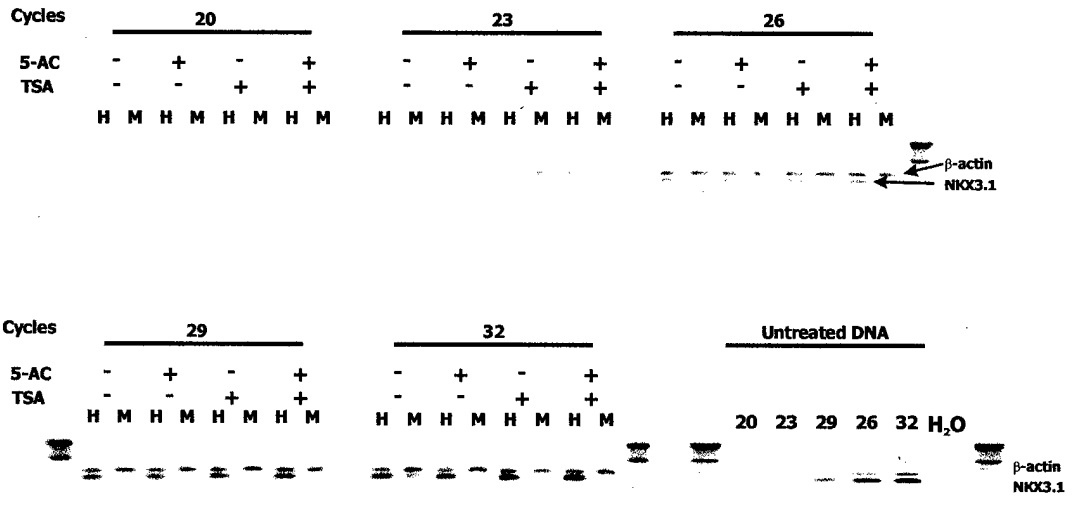
Since we saw no effect of 5-AC and TSA on the expression of either *NKX3.1* mRNA or protein we also examined the target CpG sites. Figure 6 shows the methylation status of three CpG sites, -921, -903 and –47 in DU-145 cells after treatment with these two agents. We also show the analysis of CpG –47 in LNCaP cells. We found no consistent changes in methylation of these sites in response to either 5-AC or TSA.

Fig 6
Effect of 5-AC and TSA on CpG Sites in NKX3.1



Lastly, we also examined the effect of 5-AC and TSA on the cleavage of the HpaII/MspI site at position 872 of the *NKX3.1* gene. We found that there was no effect of these agents on the cleavage of CCGG at 872 by the two restriction enzymes (Figure 7).

Fig 7
Effect of 5-AC and TSA on HpaII/MspI Cleavage at CpG 872



Key Research Accomplishments

1. Identification of methylation target sites for NKX3.1 in human prostate cancer tissues.

2. Demonstration that NKX3.1 is hypermethylated in human prostate cancer and that methylation correlates with NKX3.1 expression in primary prostate cancer cells.

Reportable Outcomes

1. Identification of methylation target sites for NKX3.1 in human prostate cancer tissues.

2. Demonstration that NKX3.1 is hypermethylated in human prostate cancer and that methylation correlates with NKX3.1 expression in primary prostate cancer cells.

Paper submitted:
Ekatherine Asatiani, Wen-Xin Huang, Antai Wang, Elizabeth Rodriguez, Luciane R. Cavalli, Bassem R. Haddad, and Edward P. Gelmann. Gene deletion and methylation reduce suppressor protein NKX3.1 expression in primary human prostate cancer.

The data presented in this report are being prepared for publication.

Conclusions

1. The DNA spanning –1200 to 1100 of the *NKX3.1* gene is highly GC rich. A select number of CpG sites are targets for methylation.

2. In vitro, methylation at these sites is not affected by 5-azacytidine or trichostatin A.

3. In vivo at least three sites in the *NKX3.1* promoter are targets for methylation and hypermethylation in cancer cells.
4. Hypermethylation correlates with reduced expression of NKX3.1 in cancers.

5. Hypermethylation appears to cooperate with gene deletion to reduce NKX3.1 expression in early stages of prostate cancer.

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